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Pre-treatment of Flowers Extract of Abelmoschus manihot Medicus on Ischemia/Reperfusion Renal Injury in Rats

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ABSTRACT

***Background and Aim:** The purpose of this study is to provide a basic data with the use of flowers extract of Abelmoschus manihot (Linnaeus) Medicus (Malvaceae) (**AM**) as a herbal agent against renal oxidative stress. **Materials and Methods:** Pre-treatment effect of flowers extract of **AM** in rat with renal ischemia/reperfusion(**I/R**) injury was investigated. Renal **I/R** injury in rat were induced by clamping of the left and right renal arteries for 45 min followed by 24 h of reperfusion after administration of flowers extract of **AM** for 30 days. The effect of flowers extract of **AM** was evaluated through the measurement of renal function and the relevant parameters of oxidative stress. **Results:** Renal **I/R** injury led to renal dysfunction as evidenced by higher serum BUN and creatinine along with increase in oxidative stress in renal tissues compared with Sham group. Pre-treatment of flowers extract of **AM** decreased serum BUN, creatinine, and renal MDA levels, and increased SOD, CAT and GSH-PX activities in the kidney. **Conclusion:** Collectively, these data indicated that flowers extract of **AM** could be used as a safe and natural antioxidant for the protection of oxidative stress in kidney.*

Keywords: Abelmoschus manihot (Linnaeus) Medicus (Malvaceae) (AM), Ischemia/Reperfusion (I/R), Oxidative stress.

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INTRODUCTION

Renal ischemia/reperfusion(I/R) injury is the most common cause of acute kidney injury (AKI), which frequent occurrence in critically ill patients. AKI leads to adverse outcomes in hospitalized patients including prolonged length of stay, higher health care costs, and increased mortality. [1] Clinically, AKI may occur as a result of several conditions and events that can lead to renal ischemia/ reperfusion (I/R) injury, such as sepsis, burns, aortic aneurysm, cardiac surgery, contrast agents, and trauma as well as post-renal transplantation, mercury poisoning, partial nephrectomy,renal artery angioplasty, and other urological conditions. [2]The mechanisms contributing to the pathophysiology of renal I/R injury are complicated and include a variety of signaling pathways that are driven by the interplay of inflammatory cytokines/chemokines, oxidative stress, reactive oxygen species (ROS), and apoptosis-related factors. [3,4,5]The production of ROS during the reperfusion period has been suggested to play a key role for uncontrolled oxidative stress, and increased ROS can also drive the inflammatory cascade. [6]

Additionally, cell apoptosis, one of the most severe outcomes of renal I/R injury, determines the level of renal destruction. Plants provide an abundant source of biologically active molecules that have played critical roles in pharmacology. The flowers of Abelmoschus manihot (Linnaeus) Medicus (Malvaceae) (**AM**) have been used in DPR Korea for a long time as a traditional KORYO medicine for the treatment of many kinds of diseases including kidney disese as well as in China and Southeast Asia. **AM**, also known as Aibika, belongs to the family of Malvaceae. [7] In many countries various functional foods have been developed from roots, stems, and leaves of this plant [8,9] and the flowers are an important herbal medicine for the treatment of chronic renal disease [10,11] , diabetic nephropathy [12], oral ulcers[13] and burns. However, the medical benifits of flowers of **AM** for the cure of several diseases is well known, but its effect to inhibit the oxidative stress in kidney induced by **I/R** mechanism has not yet been investigated in experimental studies.

MATERIALS AND METHODS

Preparation of Flowers Extract of AM

Flowers samples of **AM** collected from Sunan district, the suburban area of Pyongyang, in the spring season (April to May) were authenticated by National botanical institute and extracted by Traditional Medicine Centre in DPR of Korea. The extract used in this study was prepared using the traditional ethanolic method. Briefly, 3 kg of dry flowers including pollen were immersed in 3 L of 90% ethanol with intermittent shaking for 24 h, and then refluxed for 3 h by heating. The filtrate was evaporated below 45 ° C under reduced pressure. The residue (yield: 4.7%) was designated as an alcoholic extract. The extract was quantified by a HPLC assay to contain main components: total flavonoid at 5.4%.

Animals

Male adult Wistar rats (11-12-week-old, 250~300g) were provided by Laboratory Animal Centre of Pyongyang University of Medical Sciences and adapted in a lab environment before experiments for a week. 50 rats were randomly chosen and during the experiment feed and water were available to rats at any time. The temperature was maintained at 20±2℃ and the humidity was 55%. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Basic Medicine, Pyongyang University of Medical Sciences.

Pre-treatment of Flowers Extract of AM through Diet

After 2 weeks of acclimation, the rats were randomly divided into 5 groups (n=10):

Sham-operated group (SG): rats fed the control diet only

Model group (MG): rats fed the control diet only
Extract group-1(EG-1): rats fed a diet containing 30mg/kg extract
Extract group-2(EG-2): rats fed a diet containing 60mg/kg extract
Extract group-3(EG-3): rats fed a diet containing 90mg/kg extract
The animals in each group were fed their experimental diets daily for 30 days. Next day rats were operated upon for I/R, except Sham-animals.

I/R renal injury model

Rats were submitted to I/R renal injury model surgery as previously described. [14]

1) Mechanical method

Briefly, rats were anesthetized with Ketamine. A midline incision was made and both left and right renal pedicles were cross-clamped. After 45 min of bilateral renal ischemia, the occlusion clips were removed, and the abdominal wall was closed in two layers, and the animals were placed in single cages, warmed by indirect light, until complete recovery from anesthesia. The same procedure was performed in the sham-operated group without the bilateral clamping process.

2) Chemical method

Rats were oral administrated with $HgCl_2$ with a dose of 18mg/kg(mercury content:13mg/kg) once every day for 7 days.

Antioxidant Enzyme Activity Estimation in renal tissue

The left and right kidneys were removed under fully maintained anesthesia. After removal, the kidneys were homogenized (10000rpm, 20s) with a Teflon glass tissue homogenizer (Remi, India) in cold normal saline. The homogenate (10%) was centrifuged at 3000rpm for 20min at 4°C, and total supernatant was used for assays. Renal tissue superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity, catalase (CAT) activity, glutathione (GSH) and malondialdehyde (MDA) contents were determined with the following methods. SOD was assayed according to the previous method. [15] To 0.5mL of tissue homogenate supernatant, 0.5mL of 0.6mM EDTA solution and 1mL of 0.1M carbonate-bicarbonate (pH-10.2) buffer were added. The reaction was initiated by the addition of 0.5mL of 1.8mM epinephrine (freshly prepared) and the increase in absorbance at 480nm was measured. GSH-Px was assayed by the previous method with slight modification. [16]

The reaction mixture consisted of 0.2mL of 0.8mM EDTA, 0.1mL of 10mM sodium azide, 0.1mL of 2.5mM H2O2, 0.2mL of GSH, 0.4mL of 0.4M phosphate buffer pH 7.0, and 0.2mL of tissue homogenate supernatant and was incubated at 37.8°C for 10min. The reaction was arrested by the addition of 0.5mL of 10% Trichloroacetic acid (TCA) and the tubes were centrifuged at 2000rpm. To the supernatant, 3.0mL of 0.3mM disodium hydrogen phosphate and 1.0mL of 0.04% dithionitrobenzoic acid (DTNB) were added and the colour developed was read at 420nm immediately. The activity of GSH-Px was expressed as μ moles of glutathione oxidized/min per mg of protein. CAT was assayed by the following method. To 1.2mL of 50mM phosphate buffer pH 7.0, 0.2mL of tissue homogenate supernatant was added and reaction was started by the addition of 1.0mL of 30mM H_2O_2 solution. The decrease in absorbance was measured at 240nm at 30s intervals for 3min. The enzyme blank was run simultaneously with 1.0mL of distilled water instead of hydrogen peroxide.

GSH Estimation in renal tissue

GSH content in the supernatant was measured by reaction with DTNB. To 0.1mL of tissue homogenate supernatant, 2.0mL of 0.6mM DTNB and 0.2M phosphate buffer (pH 8.0) were added to make up to a final volume of 4.0mL. The absorbance was read at 412nm against a blank containing TCA instead of sample. A series of standards treated in a

similar was also were run to determine the glutathione content. 5-Sulphosalicylic acid was used to prevent oxidation of glutathione. The amount of glutathione was expressed as μ mol/mg of tissue.

MDA Estimation in renal tissue

MDA was determined by the thiobarbituric acid (TBA) method, based on its reaction with TBA to form thiobarbituric acid-reactive substances (TBARS). Determination of TBARS in renal tissue was measured as described previously with slight modification. To 0.2 ml of tissue homogenate supernatant, 0.2 mL of 8.1% sodium lauryl sulphate and 1.5 ml of 20% acetic acid solution (pH adjusted to 3.5 with sodium hydroxide) were added.

Then 1.5 ml of 0.8% aqueous solution of TBA was added. The mixture was made up to 40mL with distilled water and heated in a water bath at 95°C for 60 min. In cooling water 1.0 ml of distilled water and 5.0 ml mixture of n-butanol and pyridine (15:1 v/v) were added. After centrifugation at 4000 rpm for 10 min. Absorbance of the organic layer was read at 532 nm. The level of MDA was expressed as nmol/mg of tissue. Protein concentration of renal tissue was measured using the method of Lowry and others, [17] using bovine serum albumin as standard.

Assessment of renal function

Serum supernatants were stored at −80 °C until laboratory analysis. Samples were thawed before the study. Serum BUN and creatinine analyses were performed with autoanalyzers.

STATISTICAL ANALYSIS

Quantitative data are reported as mean ± standard error of the mean. Statistical differences in basal characteristics between the groups were calculated by one-way analysis of variance and t-test for continuous variables. $P < 0.05$ or $P < 0.01$ was considered statistically significant. All statistical analyses were performed using the SPSS 16.0 software.

RESULTS

As shown in **(Table 1)**, The activities of SOD, GSH-Px and CAT in renal tissue of MG rats significantly decreased compared to SG rats ($p < 0.01$, $p < 0.01$ and $p < 0.01$, respectively). Pre-treatment of extract increased activities of all enzymes in dose-dependent manners.

Table 1. Changes of antioxidant enzyme activity in renal tissue.

Groups	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)
SG	**3.3±0.4**	**2.7±0.3**	**10.3±0.7**
MG	**1.6±0.2$^{\Delta\Delta}$**	**1.1±0.1$^{\Delta\Delta}$**	**4.4±0.3$^{\Delta\Delta}$**
EG-1	**2.1±0.2**	**1.8±0.3***	**5.1±0.5**
EG-2	**2.8±0.4***	**2.3±0.3****	**8.2±0.3****
EG-3	**2.9±0.3***	**2.3±0.2****	**8.5±0.5****

Each value represents the mean ± SEM of 10 rats per group. *p<0.05, **p<0.01 as compared with MG (Model group). $^{\Delta\Delta}$p<0.05 as compared with SG(Sham-operated group).

In MG rats, there were significant changes in GSH and MDA level compared with SG rats. ($p < 0.01$ and $p < 0.01$, respectively) But pre-treatment of extract significantly improved these changes compares with MG. Improved effects were in dose-dependent manners in **(Table 2)**.

Table 2. Changes of GSH and MDA level in renal tissue.

Groups	GSH (µmol/mg Protein)	MDA (nmol/mg Protein)
SG	32.5±3.1	2.5±0.2
MG	13.4±1.8$^{\Delta\Delta}$	6.8±0.5$^{\Delta\Delta}$
EG-1	22.2±2.3**	4.3±0.1**
EG-2	25.5±2.7**	3.8±0.3**
EG-3	28.1±1.9**	3.5±0.3**

Each value represents the mean ± SEM of 10 rats per group. **p<0.01 as compared with MG (Model group). $^{\Delta\Delta}$p<0.05 as compared with SG(Sham-operated group).

The serum BUN and creatinine levels were increased in MG rats compared with those in SG rats. ($p < 0.01$ and $p < 0.01$, respectively). However, in rats pre-treated with extract, the serum levels of BUN and creatinine were significantly reduced, unlike those in MG rats in **(Table 3)**.

Table 3. Changes of BUN and Creatinine in serum.

Groups	BUN (mg/dL)	Creatinine (mg/dL)
SG	24.1 ± 1.7	0.37 ± 0.02
MG	86.7 ± 4.3$^{\Delta\Delta}$	1.48 ± 0.04$^{\Delta\Delta}$
EG-1	60.3 ± 2.5**	0.81± 0.01**
EG-2	57.9 ± 2.1**	0.66± 0.03**
EG-3	51.6 ± 2.8**	0.62± 0.01**

Each value represents the mean ± SEM of 10 rats per group. **p<0.01 as compared with MG (Model group). $^{\Delta\Delta}$p<0.05 as compared with SG(Sham-operated group).

DISCUSSION AND CONCLUSIONS

Recent evidence has indicated that the main causes of renal I/R injury include increased production of renal oxygen-free radicals, overload of intracellular calcium, and abnormal energy and hormone metabolism. [18] The damaging effect of ROS on renal tissues has been considered crucial in these complex pathophysiological processes. [19] Oxygen radicals produced after ischemia/reperfusion can cause lipid peroxidation and destroy cell and organelle membranes, thereby disrupting the tissue structure and function. [20,21] In terms of inflammation, hypoxic and anoxic cell injuries occur in the renal tissue after ischemia, leading to the local robust synthesis of inflammatory cytokines. These cytokines can initiate defensive physiological activities to isolate and inhibit tissue damage, or further aggravate organ damage and dysfunction by inducing free radicals to produce and recruit inflammatory cells. [22] Therefore, it is important to control oxidative stress and inflammation in the early stage of renal I/R injury. Abelmoschus manihot (Linnaeus) Medicus (Malvaceae) (**AM**), the main medicinal part of this plant, have been used in clinical practice to treat chronic kidney disease (CKD), inflammatory diseases, oral ulcers, and burns for hundreds of years in our country. However, there have been no studies on flowers of **AM** to treat renal I/R injury. In this study, we attempted to examine the pre-treating effect of flowers extract of AM on renal injury by I/R in rats so as to provide data available in clinical practice. Oxygen radicals cause the peroxidation of polyunsaturated fatty acids in the biofilm, and the decomposition products of lipid hydroperoxide, causing cell damage. As major antioxidant enzymes, SOD, CAT and GSH-Px, and GSH serve a crucial role in scavenging oxygen radicals, the activity of

which can reflect the ability of scavenging oxygen free radicals and the ability of the kidney to resist lipid peroxidation. The MDA content can reflect the content of oxygen free radicals, the level of lipid peroxidation, and the degree of damage of oxygen free radicals to the kidney tissue. [23,24] The present study revealed that the MDA level was decreased, while activity of antioxidant enzymes (SOD, GSH-Px and CAT) and GSH level were increased, in the renal tissues in pre-treatment of extract, demonstrating a relatively mild oxidative injury. **(Table 1~3)** These results imply that the protective role of flowers of **AM** against bilateral I/R injury may be associated with the inhibition of free radical and oxidant formation. Additionally, pre-treatment of extract significantly improved parameters such as serum BUN and creatinine associated with renal function, as shown in **(Table 3)**. In summary, protective effect of flowers of **AM** against I/R-induced renal injury by inhibiting oxidative stress in rats. A more detailed study is currently under way to explore several mechanisms underlying the protective role of flowers of **AM** in renal I/R injury.

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Conflict of interest

The authors have nothing to disclose.

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